Review Paper:

Bioactive phytochemicals in *Plumeria* sp.: extraction, quantification and analysis

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Abstract

Demand for new and novel natural compounds has intensified the development of plant-derived compounds known as bioactives that either promote health or are toxic when ingested. Plumeria sp. provides a rich source of bioactive phytochemicals like alkaloids, terpenes , terpenoids and phenolic compounds. The use of bioactive compounds in different commercial sectors such as pharmaceutical, food and chemical industries signifies the need of the most appropriate and standard method to extract these active components from plant materials. Enhanced release of these bioactives from plant cells by cell disruption and extraction through the cell wall can be optimized using conventional methods along with numerous new methods which have been established but till now no single method is regarded as standard for extracting bioactive compounds from plants.

The efficiencies of conventional and non-conventional extraction methods mostly depend on the critical input parameters; understanding the nature of plant matrix chemistry of bioactive compounds and scientific expertise. This review is aimed to discuss different extraction techniques along with their basic mechanism for extracting bioactive compounds from Plumeria sp. including their quantification and analysis.

Keywords: *Plumeria* sp., Phytochemicals, Bioactive compounds, Secondary metabolites

Introduction

Plants of genus *Plumeria* (*Apocynaceae*) have their origin from Central America and reputed medicinal properties. The genus consists of eight species growing in tropical and sub-tropical regions of the world[14,103]. Two species namely *Plumeria rubra* and *Plumeria obtusa* are found in Pakistan which are grown for ornamental purposes[79]. Various species of this genus are used as medicine to cure diarrhea, gonorrhea, syphilis, veneral sores and leprosy[81].

The members of this genus possess anti-inflammatory, diuretic, emmenagogue, febrifuge, purgative and are used as tonic and expectorant[30]. The grandines A–C, phoebegrandine B, and fulvoplumeirin, constituents of *Plumeria acutifolia* are used as antibacterial agent[3,37]. The aqueous extract of *P. rubra* showed antimicrobial[34], anti-inflammatory activities[21] and was used for the treatment of respiratory ailments[9,28].

Plumericin, an iridoid isolated from *P. rubra*is was used as antimicrobial agent[60]. Understanding the impact of processing on *Plumeria*, phytochemicals allows us to develop improved processes with higher retention of bioactive compounds in processed products.

Classification and synthesis of bioactive compounds

Classification of bioactive compounds in different categories is still inconsistent, rather it depends upon the target of the particular classification.

According to Croteau et al[17], bioactive compounds of plants are divided into three main categories: (a) terpenes and terpenoids (approximately 25,000 types), (b) alkaloids (approximately 12,000 types) and (c) phenolic compounds (approximately 8000 types).General structures of different categories of bioactive compounds are given in fig. 1.

The majority of bioactive compounds belong to one of a number of families, each of which has particular structural characteristics arising from the way in which they are built up in nature (biosynthesis).There are four major pathways for synthesis of bioactive compounds or secondary metabolites: (1) Shikimic acid pathway,(2) Malonic acid pathway, (3) Mevalonic acid pathway and (4) Non-mevalonate (MEP) pathway[97].

Alkaloids are produced by aromatic amino acids (from shikimic acidpathway) and by aliphatic amino acids (tricarboxylic acid cycle). Phenolic compounds are synthesized through malonic acid pathway and shikimic acid pathway. Through MEP pathway and mevalonic acid pathway, terpenes are produced. Simplified illustrations of different pathways for the production of three major groups of plant bioactive compounds are shown in fig. 2.

Extraction of bioactive compounds in *Plumeria*

Optimized extraction of bioactive phytochemicals is of significant importance to precisely quantify the phytochemical content in plant products. The extraction techniques for *Plumeria*sp. are shown by a flow chart in fig. 3.

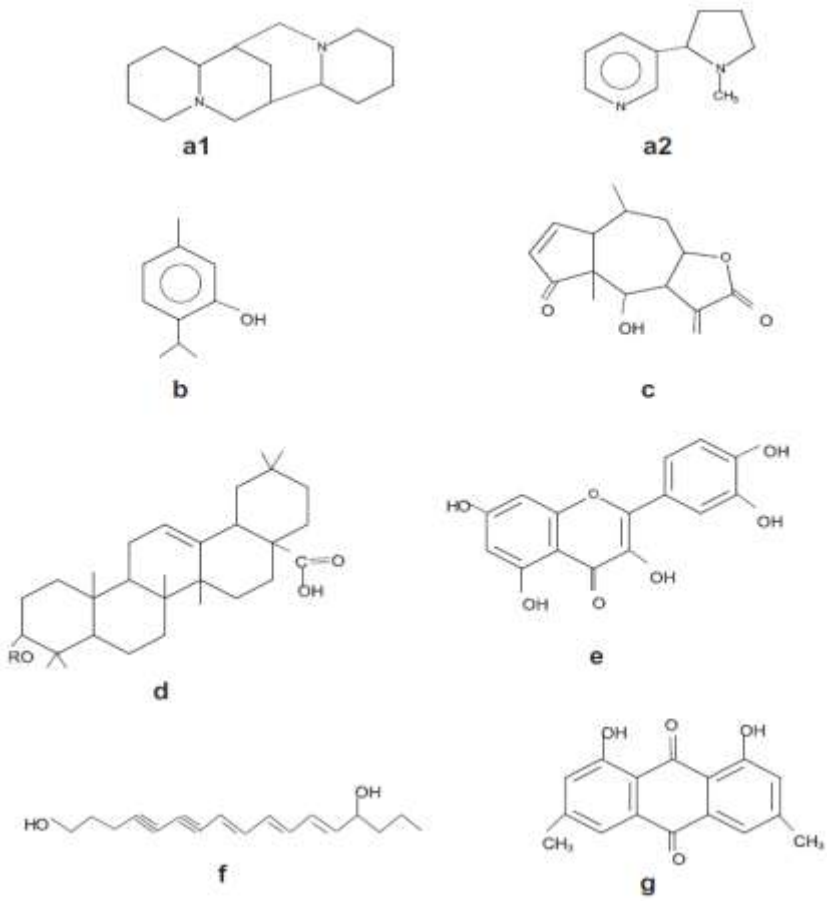

Fig. 1: General structures of different categories of plant bioactive compounds: (a1 and a2) alkaloids, (b) monoterpenes, (c) sesqueterpenes, triterpenes, saponins, (d) steroid, (e) flavonoids, (f) polyacetylenes, (g) polyketides[102]

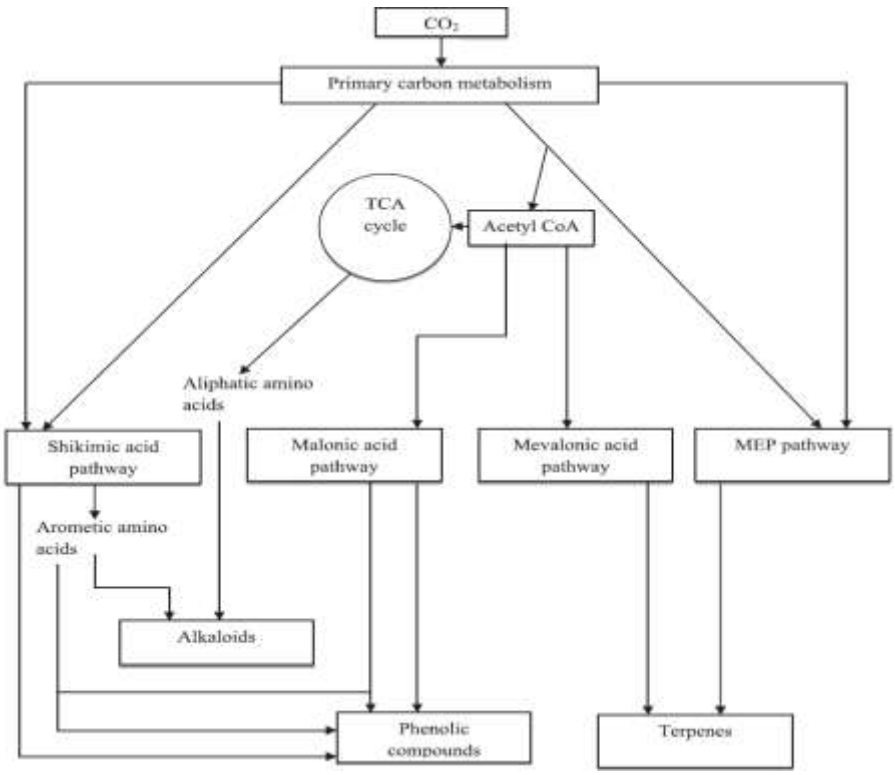

Fig. 2: A simplified view of pathways for production of three major groups of plant bioactive compounds (adapted from Tiaz and Zeiger[97])

Conventional extraction techniques: Bioactive compounds from *Plumeria* sp. can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. The existing classical techniques are:(1) Soxhlet extraction, (2) Maceration and (3) Hydrodistillation.

Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet[93]. It was designed mainly for extraction of lipid but now it is not limited for this only. The Soxhlet extraction has widely been used for extracting important bioactive compounds from various natural sources. Maceration is a popular and inexpensive way to get essential oils and bioactive compounds. The solvent used is named as menstruum.

Occasional shaking in maceration facilitates extraction by two ways; (a) increases diffusion, (b) removes concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield.

Hydrodistillation is a traditional method for extraction of bioactive compounds and essential oils from plants. Organic solvents are not involved before dehydration of plant materials. There are three types of hydrodistillation: water distillation, water and steam distillation and direct steam distillation[99].

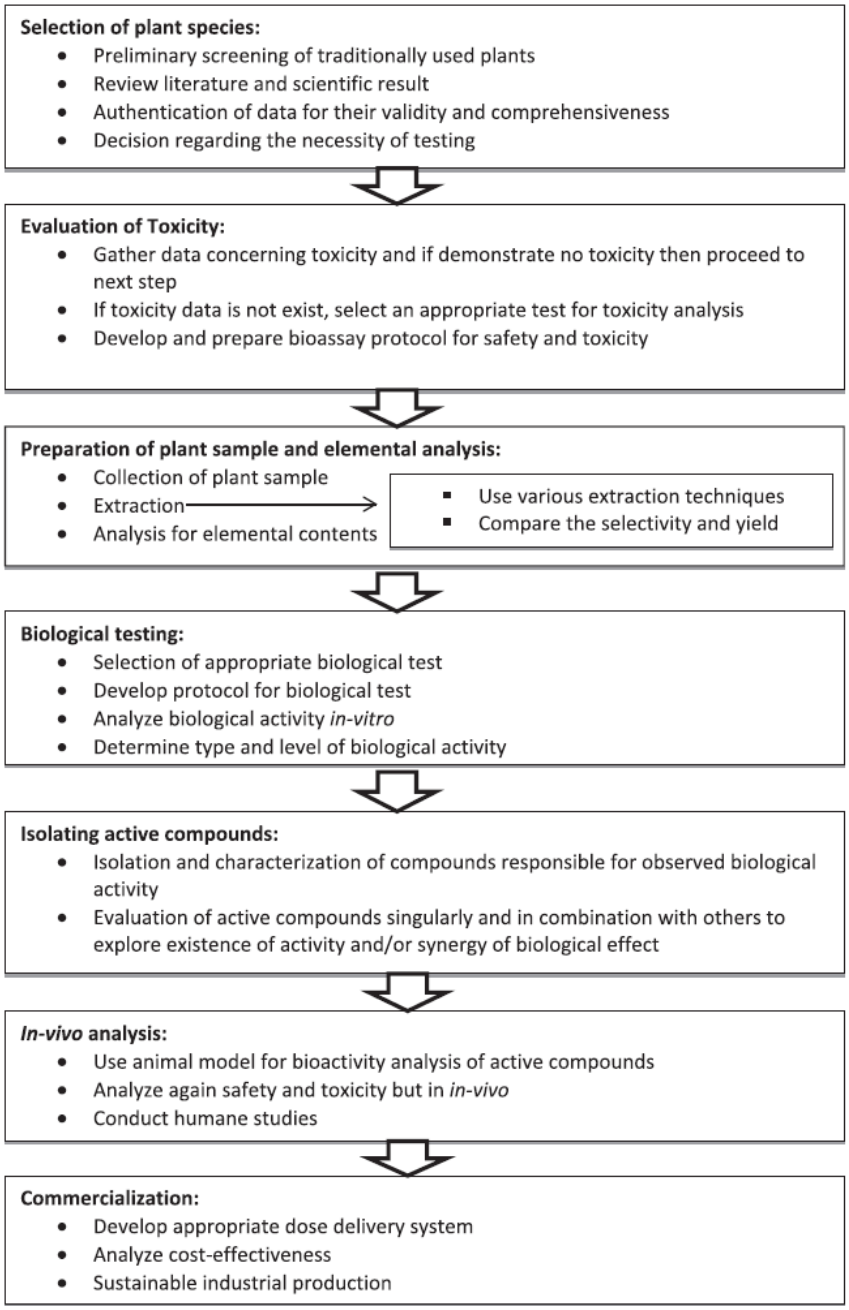

Fig. 3: The flow chart of medicinal plant study and position of extraction techniques (adapted from Farnsworth et al[26])

In hydrodistillation, first, the plant materials are packed in a still compartment; second, water is added in sufficient amount and then brought to boil. Alternatively, direct steam is injected into the plant sample. Hot water and steam act as the main influential factors to free bioactive compounds of plant tissue. Indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from condenser to a separator, where oil and bioactive compounds separate automatically from the water[90]. Hydrodistillation involves three main physicochemical processes; Hydrodiffusion, hydrolysis and decomposition by heat. At a high extraction temperature, some volatile components may be lost. This drawback limits its use for thermolabile compound extraction. Some examples of bioactive compound extracted using different solvents are given in table 1.

Non-conventional extraction techniques: The major challenges of conventional extraction are longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds[64]. To overcome these limitations of conventional extraction methods, new and promising extraction echniques are introduced. These techniques are referred as non-conventional extraction techniques. Some of the most promising techniques are ultrasound assisted extraction, pulsed electric field assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and pressurized liquid extraction (Table 2). Some of these techniques are considered as‘‘green techniques’’ as they comply with standards set by Environmental Protection Agency, USA (http://www.epa.gov/greenchemistry/pubs/about_gc.html).

Table 1
Example of some extracted bioactive compounds by different solvents (adapted from Cowan[15]).

Water	Ethanol	Methanol	Chloroform	Dichloromethanol	Ether	Acetone
Anthocyanins Tannins Saponins Terpenoids	Tannins Polyphenols Flavonol Terpenoids Alkaloids	Anthocyanin Terpenoids Saponins Tannins Flavones Polyphenols	Terpenoids Flavonoids	Terpenoids	Alkaloids Terpenoids	Flavonoids

Table 2
Methods used for extraction of bioactive phytochemicals from *Plumeria*

S. N.	Extraction	Photochemical	Results	Changes in levels of phenolics
1	Conventional solvent extraction	TPC	Different solvents showed different efficacies for extraction of phenolic compounds. The extract produced with 60% v/v acetone had the highest content of total phenols.[68]	+
2	Ultrasonic assisted extraction (UAE)	Phenolics-rich heteroxylans	UAE represents a shortening of the process by about 60% and lower consumption of the NaOH.[45]	+
3	Microwave assisted extraction (MAE)	TPC	There was little difference of levels of phenolic acids between heating with microwave irradiation and the same conditions using a regular water bath as the heat source.[52]	*
4	Pressurized liquid extraction (PLE)	Phenolic acid	The production of vanillin from crop residues containing greater levels of ferulic acid such as corn bran and sugar-beet pulp with PLE may be practical and cost-effective.[8]	+
5	Supercritical fluid extraction (SFE)	Phenolic acid	Using an optimized solid-phase extraction (SPE) method a range of 84% to 106% recovery rate was obtained for phenolic acids in cereals including wheat.[5]	+
6	Supercritical fluid extraction (SFE)	Tocopherols, tocotrienols and carotenoids	Encapsulation of SC-CO_2 extracted wheat bran oil in alginate beads resulted in a protective effect on nutritionally important compounds such as tocopherols, tocotrienols, lutein, zeaxanthin, and β-carotene.[42]	+
	Solid-phase	Phenolic acid	Using an optimized solid-phase extraction (SPE)	*

7	extraction (SPE)		method a range of 84% to 106% recovery rate was obtained for phenolic acids in cereals including wheat.[31]	
8	Supercritical fluid extraction (SFE)	Tocopherols and tocotrienols	Tocopherol yield was 0.33 mg tocopherol/g germ at 405 bar, 60 °C and 10 min).[23]	+
9	Supercritical fluid extraction (SFE)	Tocopherols and tocotrienols	Optimum value predicted by RSM for the concentration of natural vitamin E was 2307 mg/100g.[35]	*
10	Solid-phase extraction (SPE)	Polyphenols	Enzymatic extraction seems to be more efficient than organic solvents for phenol bioaccessibility in wheat bread.[49]	+
11	Conventional solvent extraction	Alkylresorcinols	Ethyl acetate used as solvent at room temperature under periodic hand shaking for 48 h. After extraction the solvent layer was filtered and evaporated to dryness before analysis.[43]	*
12	Conventional solvent extraction	Alkylresorcinols	Acetone used as extraction solvent in a 1:40 (w/v) ratio followed by continuous stirring (Stirrer-VWR, Corning®, VMS-C4) at room temperature for 24h.[95]	*
13	Conventional solvent extraction	Carotenoids and tocopherols	Butanol method extraction is best for carotenoid extraction, while hot saponification and the butanol protocols have better extraction efficiency than the other extraction method for tocopherols.[33]	*
14	Supercritical fluid extraction (SFE)	Alkyl resorcinols	CO_2 desired operating pressure: 40.0 MPa, extracting temperature of 40–80 °C, and the carbon dioxide flow rate of 1.5 ± 0.3 g/min was used for the extraction and the results were compared with conventional solvent extraction. The extraction yield was higher for polar organic solvents than for SC-CO_2.[53]	*
15	Ultrasonic assisted extraction (UAE)	Alkyl resorcinols	Dichloromethane was used under ultra-sound-assisted extraction method and the samples were sonicated for 15 s at 50% amplitude. During ultra sonication, samples were cooled in an ice bath to avoid sample heating. The duration of analytical extraction was shortened from more than 1 h to only 45 s as compared to previous methods. In addition, sample weight and solvent use were significantly reduced.[12]	+
16	Supercritical fluid extraction (SFE)	Tocopherols	Pressure of 4000–5000 psi, the extracting temperature of 40–45 °C, and the carbon dioxide flow rate of 2.0 mL/ min for 90 min were their optimal extraction conditions to extract tocopherols from wheat germ.[31]	*
17	Accelerated solvent extraction (ASE)	Benzoxazinoids	Dionex ASE 350 Accelerated Solvent Extractor was used with the extraction solvent containing 19% water, 80% methanol and 1% acetic acid. The extraction was carried out at 80 °C.[76]	*
18	Accelerated solvent extraction (ASE)	Phytosterols	Different extraction solvents with different temperature were used to optimize the extraction condition using ASE.[22]	*
19	Acid/ base hydrolyses	Phytosterols	The phytosterols were extracted by using acid and base hydrolyses of the samples followed by silica gel column purification prior to the analysis.[73]	*
20	Hot acetone under reflux	Steryl ferulates	Hot acetone under reflux followed by base–acid for the purification of the extract.[73]	*
21	Alkaline hydrolysis / enzyme digest	Lignans	The alkaline hydrolyzed samples were column purified using SPE column.[18]	*

These include less hazardous chemical synthesis, designing safer chemicals, safe solvents auxiliaries, design for energy efficiency, use of renewable feedstock, reduced derivatives, catalysis, design to prevent degradation, atom economy and time analysis for pollution prevention and inherently safer chemistry for the prevention of accident.

Ultrasound-assisted extraction (UAE): The extraction mechanism by ultrasound involves two main types of physical phenomena, (a) the diffusion across the cell wall and (b) rinsing the contents of cell after breaking the walls. Moisture content of sample, milling degree, particle size and solvent are very important factors for obtaining efficient and effective extraction. Furthermore, temperature, pressure, frequency and time of sonication are the governing factors for the action of ultrasound.

The advantages of UAE include decrease in extraction time, energy and use of solvent. Ultrasound energy for extraction also facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective extraction, reduced equipment size, faster response to process extraction control, quick start-up, increased production and eliminates process steps[11].

UAE seemed to be an effective extraction technique for bioactive compound extraction from herbal plants. Ionic liquid based UAE technique proved to have high efficiency and shorter extraction time than conventional extraction methods[106].

Pulsed-electric field extraction (PEF): The principle of PEF is to destroy cell membrane structure for increasing extraction. During suspension of a living cell in electric field, an electric potential passes through the membrane of that cell. Based on the dipole nature of membrane molecules, electric potential separates molecules according to their charge in the cell membrane. After exceeding a critical value of approximately 1 V of transmembrane potential, repulsion occurs between the charge carrying molecules that form pores in weak areas of the membrane and cause severe increase of permeability[7]. Usually, a simple circuit with exponential decay pulses is used for PEF treatment of plant materials. The efficiency of PEF treatment strictly depends on the process parameters including field strength, specific energy input, pulse number, treatment temperature and properties of the materials to be treated[40].

PEF has been applied to get better release of intracellular compounds from plant tissue with the help of increasing cell membrane permeability[98]. PEF treatment at a moderate electric field (500 and 1000 V/cm; for10^{-4}–10^{-2} s) is found to damage cell membrane of plant tissue with small temperature increase[27,58]. Due to this reason, PEF can reduce the degradation of heat sensitive compounds[2]. PEF is also applicable on plant materials as a pretreatment process prior to conventional extraction to lesser extraction effort[61].

Enzyme-assisted extraction (EAE): Enzymatic pre-treatment has been considered as a novel and an effective way to release bounded compounds and increase overall yield[86]. The addition of specific enzymes like cellulase, α-amylase, and pectinase during extraction increases recovery by breaking the cell wall and hydrolyzing the structural polysaccharides and lipid bodies[86,92].

There are two approaches for enzyme-assisted extraction: (1) enzyme-assisted aqueous extraction (EAAE) and (2) enzyme-assisted cold pressing (EACP)[57]. Usually, EAAE methods have been developed mainly for the extraction of oils from various seeds[38,86,88]. In EACP technique, enzymes are used to hydrolyze the seed cell wall because in this system polysaccharide-protein colloid is not available which is obvious in EAAE[13]. Various factors including enzyme composition and concentration, particle size of plant materials, solid to water ratio, and hydrolysis time are recognized as key factors for extraction[74].

Dominguez et al[20] reported that the moisture content of plant materials is also an important factor for enzymatic hydrolysis. The EAE is recognized as eco-friendly technology for extraction of bioactive compounds and oil because it uses water as solvent instead of organic chemicals[82].

Microwave assisted extraction (MAE): The microwave-assisted extraction is also considered as a novel method for extracting soluble products into a fluid from a wide range of materials using microwave energy[77].

The extraction mechanism of microwave assisted extraction is supposed to involve three sequential steps described by Alupului[4]: first, separation of solutes from active sites of sample matrix under increased temperature and pressure; second, diffusion of solvent across sample matrix; third, release of solutes from sample matrix to solvent. Several advantages of MAE have been described by Cravottoa et al[16] such as faster heating for the extraction of bioactive substances from plant materials; reduced thermal gradients; reduced equipment size and increased extract yield.

MAE can extract bioactive compounds more rapidly and a better healing is possible than conventional extraction processes. It is a selective technique to extract organic and organometallic compounds that are more intact. MAE is also recognized as a green technology because it reduces the use of organic solvent[4].

MAE is faster and easier method in comparison to conventional extraction processes.

Pressurized liquid extraction (PLE) / accelerated fluid extraction (ASE) / enhanced solvent extraction (ESE) / high pressure solvent extraction (HPSE): In comparison to the traditional Soxhlet extraction, PLE was found to dramatically reduce time consumption and solvent use.

Nowadays, for extraction of polar compounds, PLE is also considered as a potential alternative technique to supercritical fluid extraction[54]. PLE is also useful for the extraction of organic pollutants from environmental matrices which are stable at high temperatures[101]. Applications of PLE technique for obtaining natural products are often available in literature[54]. Additionally, due to small amount, PLE gets broad recognition as a green extraction technique[48].

In consideration of yield, reproducibility, extraction time, and solvent consumption, PLE has been considered as an alternate to conventional methods due to faster process and lower solvent use. Luthria[66] showed that temperature, pressure, particle size, flush volume, static time, and solid-to-solvent ratio parameters have influence on the extraction of phenolic compounds by PLE.

Supercritical fluid extraction (SFE): The application of supercritical fluid for extraction purposes started with its discovery by Hannay and Hogarth[39] but the credit should also be given to Zosel[105] who presented a patent for decaffeination of coffee using SFE.

Every earthly substance has three basic states namely; solid, liquid and gas. Supercritical state is a distinctive state and can only be attained if a substance is subjected to temperature and pressure beyond its critical point. Critical point is defined as the characteristic temperature (Tc) and pressure (Pc) above which distinctive gas and liquid phases do not exist[49]. In supercritical state, the specific properties of gas and/or liquid become vanish which means supercritical fluid cannot be liquefied by modifying temperature and pressure. Supercritical fluid possesses gas-like properties of diffusion, viscosity, and surface tension and liquid-like density and solvation power. These properties make it suitable for extracting compounds in a short time with higher yields[89].

Carbon dioxide is considered as an ideal solvent for SFE. The critical temperature of CO_2 (31°C) is close to room temperature, and the low critical pressure (74 bars) offers the possibility to operate at moderate pressures, generally between 100 and 450 bar[96]. The only drawback of carbondioxide is its low polarity which makes it ideal for lipid, fat and non-polar substance, but unsuitable for most pharmaceuticals and drug samples. The limitation of low polarity of carbon dioxide has been successfully overcome by the use of chemical modifier[32,56]. Usually a small amount of modifier is considered as useful to significantly enhance the polarity of carbon dioxide.

The successful extraction of bioactive compounds from plant materials relies upon several parameters of SFE and most significantly these parameter are tunable[83]. These parameter need to be precisely controlled for maximizing benefits from this technique. The major variables influencing the extraction efficiency are temperature, pressure, particle size and moisture content of feed material, time of extraction, flow rate of CO_2, and solvent-to-feed-ratio[48,96].

The advantages of using supercritical fluids for the extraction of bioactive compounds can be understood considering following points[56]: (1) The supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent leading to more penetration to sample matrix and favorable mass transfer. Extraction time can be reduced substantially by SFE in compared with conventional methods. (2) The repeated reflux of supercritical fluid to the sample provides complete extraction. (3) The selectivity of supercritical fluid is high than liquid solvent as its solvation power can be tuned either by changing temperature and/or pressure. (4) Separation of solute from solvent in conventional extraction process can easily be bypassed by depressurization of supercritical fluid which will save time. (5) SFE is operated at room temperature, so it is an ideal method for thermo labile compound extraction. (6) In SFE, small amount of sample can be extracted and compared with solvent extraction methods which will save time for overall experiment. (7) SFE uses little amount of organic solvent and considered as environment friendly. (8) On-line coupling of SFE with chromatographic process is possible which is useful for highly volatile compounds. (9) The recycling and reuse of supercritical fluid are possible and thus minimizing waste generation. (10) SFE scale can be arranged on specific purpose from few milligram samples in laboratory to tons of sample in industries. (11) SFE process provides information regarding extraction process and mechanism which can be manipulated to optimize extraction process.

Extract preparation and yield estimation

Fresh leaves, flowers and stem bark of *Plumeria*sp. were obtained from standing living plants, cleaned and air-dried for 15 days. The dried samples were ground into smaller particles (chips) using hammer milling machine so as to increase the surface area. Five hundred grams (500 g) of the powdered *Plumeria*sp. leaf, flower and stem bark were weighed into flask and soaked with 2.5 L of 70% ethanol for 5 days with constant shaking. The ethanol extract was then filtered and concentrated using a rotary evaporator at 40 °C and yield is determined.

Yield was measured as the dry weight of the extract to the original dry weight of the sample material in percentage following the formula as in eq. 1:

$$\text{Yield estimation} = \frac{\text{Dry weight of concentrated extracts}}{\text{Weight of the ground sample}} \times 100 \quad (1)$$

Extraction of phenolic compounds: The phenolic acids have three primary states: soluble free, soluble conjugated, and insoluble bound[71]. The authors reported that most phenolic acids (~90%) occur in the insoluble bound and <9%

and <1% respectively and exist in soluble conjugated and soluble free forms. Alternatively, a simplified direct method in the presence of ascorbic acid and EDTA was used for the hydrolysis and extraction of total phenolic acids. This direct method provides marginal improvement in extraction of total phenolic acids from wide array of food matrices including *Plumeria*[62].Ultrasonic-assisted extraction (UAE) is an inexpensive and high-efficient extraction method. Simply, its mechanism depends on the collapse of bubbles that can accelerate the release of extractable compounds through the disruption of biological membranes and aids better solvent–solute interactions[100].

In the study of Hromadkova et al,[46] UAE was successfully used in the extraction of various phenolic compounds from wheat bran samples. Microwave-assisted extraction (MAE) uses microwave energy to facilitate partition target compound from plant cells into the extraction solvent. The major advantage of MAE is the shortened extraction time with lower quantity of solvent as compared to conventional extraction method[25].

Pressurized liquid extraction (PLE) is a relatively new technology used for the extraction of phytochemicals under high temperature and pressure. With PLE, application of high pressure allows extraction above the normal boiling point of the extraction solvent. The combined use of high pressures (3.3– 20.3 MPa) and high temperatures (40–200 °C) provides rapid extraction with reduced amounts of solvent (e.g. 20 min extraction with 10–50 mL of solvent using PLE provides similar or better extraction yields of phytochemicals as compared to traditional extraction method using 10–48 h and up to 200 mL of solvent)[69].

Solid-phase extraction (SPE) is widely used because it is a rapid, economical, and sensitive technique for partial purification of extracts. Different cartridges with varying numbers of sorbents can be used in SPE. Irakli et al[51] developed and validated a high performance liquid chromatography (HPLC) method for determination of free and bound phenolic acids in cereals using solid-phase extraction on Oasis HLB cartridges with aqueous methanol as a solvent.

Extraction of carotenoids and tocopherols: Supercritical fluid extraction (SFE) has been applied to the extraction of compounds as it offers several advantages namely high selectivity and less extraction time and solvent cost[41]. Durante et al[23] showed that carotenoids were relatively stable at low temperature. However, an increase in temperature resulted in decreased tocopherol concentrations due to hydroperoxide formation caused by lipid peroxidation.

Irakli et al[50] determined the carotenoids, tocopherols and tocotrienols by HPLC. The extraction method included sample saponification and clean-up by SPE using OASIS HLB SPE cartridges.

Rezaee et al[85] used dispersive liquid–liquid microextraction (DLLME) for the extraction of tocopherols and tocotrienols. The advantages of this method were reduced extraction time and comparatively less solvent usage making it cost efficient.

Extraction of miscellaneous bioactive phytochemicals: The miscellaneous group of phytochemicals includes sterols and lignans. Phytosterols can be extracted by acid and/or base hydrolysis of the sample followed by silica column purification. The extraction method for lignans used an initial alkaline hydrolysis of the sample followed by incubation with an enzyme (pomatia-glucuronidase/sulfatase). After incubation, the mixture can be purified on a SPE column. The ethanol:water solvent mixture can be used for the lignan extraction. The extracts can be acid hydrolyzed and filtered before analysis[104].

Quantification of phytochemicals of the extract

The extract can quantitatively be assessed for total content of phytochemical compounds such as alkaloids, flavonoids, phenol, saponins and tannins using the standard procedures reported by Adedeji et al.[1]

Procedures: Methanolic extract of the samples can be prepared following the method of Chan et al[10] by adding 25 mL of methanol to 0.5 g of sample contained in a covered 50 mL centrifuge tube and shaking continuously for 1 h at room temperature. The mixture can be centrifuged at 3000 rpm for 10 min, and then the supernatant can be collected and stored at -20 °C until further analysis.

Quantification of total alkaloid content (TAC): The total alkaloid contents in the samples can be measured using 1, 10-phenanthroline method described by Singh et al[91] with slight modifications. 100 mg sample powder can be extracted in 10 ml 80% ethanol. This can be centrifuged at 5000 rpm for 10 min. Supernatant obtained can be used for further estimation of total alkaloids. The reaction mixture contained 1 mL plant extract, 1 mL of 0.025 M $FeCl_3$ in 0.5 M HCl and 1 mL of 0.05 M of 1, 10-phenanthroline in ethanol. The mixture can be incubated for 30 min in hot water bath with maintained temperature of 70±2 °C. The absorbance of red coloured complex is to be measured at 510 nm against reagent blank.

Alkaloid contents can be estimated and calculated with the help of standard curve of quinine (0.1 mg/mL, 10 mg dissolved in10 mL ethanol and diluted to 100 mL with distilled water). The values can be expressed as g.100 $g^{-1}$of dry weight.

Quantification of total flavonoid content (TFC): TFC can be determined by Aluminium chloride method as reported by Kale et al.[53] Sample (0.5 mL) of extract can be dispensed into test tube, followed by 1.5mL of methanol, 0.1mL of aluminium chloride (10%), 0.1mL of 1Mpotassiumacetate and 2.8 mL of distilled water. The reaction mixture can be

mixed, allowed to stand at room temperature for 30 min, before absorbance was read at 514 nm. TFC can be expressed as quercetin equivalent (QE) in mg/g material.

Quantification of total phenolic content (TPC): The total phenolic content of sample extracts can be determined according to the Foline-Ciocalteu method used by Chan et al.[10] Briefly,300 mL of extract can be dispensed into test tube (in triplicate). To this, 1.5mL of Foline-Ciocalteu reagent (diluted 10 times with distilled water) is to be added followed by 1.2mL of Na_2CO_3 solution (7.5w/v). The reaction mixture is to be mixed, allowed to stand for 30 min at room temperature before the absorbance can be measured at 765 nm against a blank prepared by dispensing 300 mL of distilled water instead of sample extract. TPC can be expressed as Gallic acid equivalent (GAE) in mg/g material.

Quantification of total saponins content (TSC): Total saponins (TS) can be determined by the method of Hiai et al[42] as described by Makkar et al[68] with some modifications. Sample (0.5 g) can be extracted with 25 mL of 80% aqueous methanol by shaking on a mechanical shaker for 2 h, after which the contents of the tubes can be centrifuged for 10 min at 3000 rpm. In a test tube analiquot (0.25 mL) of the supernatant is to be taken to which 0.25 mLvanillin reagent (8% vanillin in ethanol) and 2.5 mL of 72% aqueous H_2SO_4 are to be added.

The reaction mixtures in the tubes are to be heated on a water bath at 60 °C for 10 min. Then tubes are to be cooled in ice for4 min and then allowed to acclimatize to room temperature. Subsequently, the absorbance can be measured in a UV/Visible spectrophotometer at 544 nm. Diosgenin can be used as a standard and the results obtained can be expressed as mg diosgenin equivalent per g of sample dry matter.

Quantification of total tannin content (TTC): Tannin content of samples can be determined according to the method of Padmaja[76] as follows: Sample (0.1 g) can be extracted with 5 mL of acidified methanol (1% HCl in methanol) at room temperature for 15 min. The mixture is to be centrifuged at 3,000 rpm for 20 min. 0.1 mL of the supernatant is to be added with 7.5 mL of distilled water, 0.5 mL of Folin-Denis reagent, 1 mL of 35% sodium carbonate solution and diluted to 10 mL with distilled water. The mixture is to be shaken well, kept at room temperature for 30 min and absorbance is to be measured at 760 nm. Blank was prepared with water instead of the sample. Tannin content can be expressed as tannic acid equivalent (TAE) in mg/g material.

Analysis of phytochemicals in *Plumeria* sp.

The analytical methods used for the determination of phytochemicals are continuously changing due to rapid advances in technology. The method used for analysis depends on the resources available and the research goals (Table 3). High performance liquid chromatography (HPLC) and gas chromatography (GC) methods have been extensively used for separation, identification, and quantification of phytochemical compounds in *Plumeria*[33,62,66].

HPLC with diode array detection (DAD) is the most popular and reliable technique and is commonly preferred over GC which frequently requires an additional derivatization step. Spectroscopic techniques including mass spectrometry (MS), nuclear magnetic resonance (NMR), and near-infrared (NIR) detections are commonly used for structural elucidation, qualitative, and quantitative analysis[73,94].

Analysis of phenolic acids: HPLC-DAD and HPLC-MS are frequently used for the analysis of phenolic acids in *Plumeria*. In previous studies, Lu et al[63] determined the insoluble and soluble ferulic acid content in other plant samples using HPLC. The authors indicated that around 99% of the ferulic acid was present in an insoluble bound form in wheat bran samples. In addition, Nicoletti et al[73] investigated the soluble free, conjugated, and insoluble bound phenolic acids in durum wheat using LC-MS. Their results showed that the total content of the three different forms of phenolic acids was in the order insoluble bound > soluble conjugated > soluble free. The authors also pointed out that ferulic acid was the predominant phenolic acid in the soluble free and insoluble bound forms whereas sinapic acid was the predominant phenolic acid in the soluble conjugated form.

Dinelli et al[19] analyzed various phenolic compounds including phenolic acids, flavonoids, coumarins, proanthocyanidins, stilbenes, and lignans from old and modern varieties using HPLC coupled with time-of flight (TOF)-MS. They concluded that some varieties may provide unique functional properties for their bioactive phytochemical content suggesting their use into a broad range of regular and special products. NMR spectroscopy is used for structural elucidation of bioactive phytochemicals. It has also been used recently for metabolomic studies.

Sun et al[94] identified and characterized *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and cinnamic acid and lignin by using NMR and NIR. In addition, minor quantities of esterified or etherified *p*-coumaric (0.06%) and ferulic acid (0.08%) were observed in the lignin preparations, suggesting that these two hydroxycinnamic acids are strongly linked to lignins.

Analysis of carotenoids and tocopherols: Tocopherols and carotenoids are also commonly analyzed by HPLC-MS and GC–MS. Hung and Hatcher[47] developed a fast and sensitive method for separation of carotenoids from plant extracts by uHPLC. The peaks of all carotenoid components are detected at 445 nm using a photodiode array detector.

Similarly, Ndolo and Beta[72] investigated the distribution of the carotenoids in endosperm, germ, and aleurone fractions of four soft wheat cultivars using HPLC. The results

indicated that the germ fraction of wheat had significant levels of lutein (2157 _g/kg) and zeaxanthin (3094 _g/kg). Lower amounts of lutein (557g/kg) and zeaxanthin (not detected) were found in the wheat endosperm. In addition, the average zeaxanthin content was 3.5 times higher in wheat germ than in yellow corn.

Beleggia et al[6] examined the metabolite profiles of plant extracts using GC and GC–MS. The authors identified tocopherols, phytosterols, amino acids as well as other metabolites using GC–MS. This method can provide a basis for developing improved plant cultivars with increased nutritional value and health benefits. The total carotenoids could also be determined as a measure of total xanthophylls by a spectrophotometric method[65].

Analysis of miscellaneous bioactive phytochemicals: Dinelli et al[19] reported phytochemical profiles of plants by HPLC-ESI-TOF-MS. In this experiment, a RP C18 analytical column with water and acetonitrile acidified with 0.5% acetic acid (v/v, mobile A and B) was used for the separation of the phytochemicals. The ESI-MS parameters (capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi with the mass range from m/z 50 to 1000) were optimized for the profiling of 70 phytochemicals including flavonoids, phenolic acids and proanthocyanidins.

Phytosterols and sterylferulates were analyzed using GC and HPLC techniques. For the phytosterol analysis, the extracts were derivatized before injecting into the GC system[75]. The individual phytosterols, sitosterol, campesterol, sitostanol, campestanol and stigmasterol were reported. HPLC[75] and HPLC-MS[36] based analysis of sterylferulates indicated that the levels of individual sterylferulates (sitosterylferulate, sitostanylferulate, and campesterylferulate) in phytochemicals were highly varied in their distribution. Lignans were analyzed by Cukelj et al[18] using GC–MS after derivatizing the extracts with pentafluoropropionic anhydride (PFPA) to improve the volatility of the metabolites.

Conclusion

The ever growing demand to extract plant bioactive compounds encourages continuous search for convenient extraction methods. The chromatography advancement and awareness about environment are two important factors for the development of most non-conventional extraction processes. However, understanding of every aspect of non-conventional extraction process is vital as most of these methods are based on different mechanism and extraction enhancement resulted from different process.

Incorporation and development of hybrid methods should also be investigated considering plant material characteristics and choice of compounds. Sufficient experimental data is still lacking in some of the existing methods. Proper choice of standard methods also influences the measurement of extraction efficiency. On the other hand, the increasing economic significance of bioactive compounds and commodities rich in these bioactive compounds may lead to find out more sophisticated extraction methods in future.

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